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Regulation of Exocytosis by Cyclin-dependent Kinase 5 via Phosphorylation of Munc18*

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Munc18a, a mammalian neuronal homologue of *Saccharomyces cerevisiae* Sec1p protein, is essential for secretion, likely as a result of its high affinity interaction with the target SNARE protein syntaxin 1a (where SNARE is derived from SNAP receptor (the soluble *N*-ethylmaleimide-sensitive fusion protein)). However, this interaction inhibits vesicle SNARE interactions with syntaxin that are required for secretory vesicles to achieve competency for membrane fusion. As such, regulation of the interaction between Munc18a and syntaxin 1a may provide an important mechanism controlling secretory responsiveness. Cyclin-dependent kinase 5 (Cdk5), a member of the Cdc2 family of cell division kinases, co-purifies with Munc18a from rat brain, interacts directly with Munc18a *in vitro,* and utilizes Munc18a as a substrate for phosphorylation. We have now demonstrated that Cdk5 is capable of phosphorylating Munc18a *in vitro* within a preformed Munc18a·syntaxin 1a heterodimer complex and that this results in the disassembly of the complex. Using site-directed mutagenesis, the Cdk5 phosphorylation site on Munc18a was identified as Thr^{574}. Stimulation of secretion from neuroendocrine cells produced a corresponding rapid translocation of cytosolic Cdk5 to a particulate fraction and an increase of Cdk5 kinase activity. Inhibition of Cdk5 with olomoucine decreased evoked norepinephrine secretion from chromaffin cells, an effect not observed with the inactive analogue iso-olomoucine. The effects of olomoucine were independent of calcium influx as evidenced by secretory inhibition in permeabilized chromaffin cells and in cells under whole-cell voltage clamp. Furthermore, transfection and expression in chromaffin cells of a neural specific Cdk5 activator, p25, led to a strong increase in nicotinic agonist-induced secretory responses. Our data suggest a model whereby Cdk5 acts to regulate Munc18a interaction with syntaxin 1a and thereby modulates the level of vesicle SNARE interaction with syntaxin 1a and secretory responsiveness.

The most generalized form of the SNARE[1] hypothesis describes a series of biochemical steps, common among diverse cell types and organisms, that mediate the trafficking of subcellular vesicles (1). In the case of regulated exocytosis, the formation and dissociation of SNARE protein complexes is essential and under spatial and temporal control (1–3). In neurons, the final stages of vesicle priming and membrane fusion leading to neurotransmitter release are also strictly Ca^{2+}-dependent (4). In addition to Ca^{2+}, there are a number of other factors that have been postulated to regulate the secretory machinery either positively or negatively. One such factor is the protein Munc18a (also termed nSec1 and rbSec1), a mammalian homologue of the *Saccharomyces cerevisiae* Sec1p protein (5–7). Munc18a is at once essential and inhibitory to secretion (8) because while Sec1p and its homologues are necessary for membrane trafficking and the final stages of protein secretion (9, 10), high affinity binding between Munc18a and syntaxin 1a also inhibits the association of vesicle SNAREs with syntaxin 1a (5–7, 11). The association of vesicle SNAREs with syntaxin is both essential (1–3) and sufficient (12) for formation of a protein core complex that mediates vesicle fusion. Genetic evidence has also established that the *Drosophila* Sec1 homologue ROP functions *in vivo* to regulate neurotransmitter release via binding to syntaxin (13). As such, any factor that regulates the interaction of Munc18a with syntaxin 1a, either by increasing their affinity or by prompting their dissociation, might be crucial to the ultimate control of the secretory process.

One candidate for this type of regulation is Cdk5, a member of the Cdc2 family of cell cycle kinases that has recently been found to co-precipitate with Munc18a from rat brain (14). Unlike the other members of this family, Cdk5 appears to be neither directly involved in the cell cycle nor activated by a cyclin (15, 16). Indeed, Cdk5 was first isolated from brain tissue as part of the Nclk (neuronal Cdc2-like kinase) complex, where it was found to be associated with a 35-kDa neural specific activator protein now termed p35 (17–19). In this capacity, Cdk5 has been demonstrated to act as a proline-directed serine/threonine kinase, phosphorylating neurofilament and tau protein at (S/T)P*X*(K/R) sites (20, 21). Moreover, mice that lack p35 or Cdk5 have been shown to suffer from severe cortical lamination defects, suggesting that the Nclk complex is also essential for proper neuronal migration and, therefore, for brain development in general (22, 23). Although Cdk5 can associate with cyclin D, it does not appear that, despite its demonstrated structural similarities to p35, cyclin D is able to fully activate Cdk5 (24). This has led to the suggestion that cyclin D exerts an indirect effect on Cdk5 by competing with p35 for binding (25). Cdk5 is further thought to differ from the other cell cycle

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[1] The abbreviations used are: SNARE, SNAP receptor; NSF, *N*-ethylmaleimide-sensitive factor; SNAP, soluble NSF attachment protein; Cdk5, cyclin-dependent kinase 5; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; ds, double-stranded; PIPES, 1,4-piperazinediethanesulfonic acid; hGH, human growth hormone; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; Cm, membrane capacitance.

kinases in that it does not appear to be directly regulated by phosphorylation. Thus, it is neither activated by CDK-activating kinase nor inhibited by Wee1 kinase, although it possesses consensus sites for both (26). However, recent work has also determined that there may be a number of regionally specific isoforms of p35 and that levels of p35 are under strict control (27). Thus, regulation of Cdk5 may be as intricate and highly specialized as that of the other members of the Cdc2 family (28).

Munc18a is a potential substrate for Cdk5 phosphorylation as it contains two of the Cdk5 consensus sequences identified from neurofilament and tau protein (9). Furthermore, the phosphorylation state of Munc18a has previously been shown to be a crucial determinant of its interaction with syntaxin 1a. When phosphorylated by protein kinase C, Munc18a has been shown to have a greatly reduced affinity for syntaxin 1a, although protein kinase C has proven ineffective at phosphorylating Munc18a already bound to syntaxin 1a (29). Recently it has been demonstrated that Cdk5 bound to its 35-kDa activator protein not only binds Munc18a but utilizes it as a substrate for phosphorylation, and that Munc18a phosphorylated in this manner has a significantly reduced affinity for syntaxin 1a (30).

The focus of the present investigation was to attempt to more completely characterize the interaction between Cdk5 and Munc18a and to establish the likelihood of a regulatory role for Cdk5 in the secretory mechanism.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—Recombinant pGEX plasmid constructs containing GST-nSec1 (rat), GST-syntaxin 1a (rat), GST-Cdk5 (human), and GST-p25 (bovine) were gifts of R. Scheller and J. Wang. *Escherichia coli* strain TG-1 was used as the host for the bacterial expression recombinant plasmids. Mouse monoclonal anti-Munc18 was obtained from Transduction Laboratories, and rabbit polyclonal anti-Cdk5 (C8) was purchased from Santa Cruz Biotechnology, Inc. Protein kinase C purified from rat brain was purchased from Calbiochem. Olomoucine and iso-olomoucine were from LC Laboratories. [γ-^{32}P]ATP was purchased from NEN Life Science Products. Sprague-Dawley rats were asphyxiated with carbon monoxide and decapitated prior to preparation of neural lobe tissue samples.

Construction of Expression Plasmids and Vectors—pGEX plasmid double-stranded DNA with the GST-nSec1 insert was purified from transformed bacteria strain (TG-1) with QIAprep Spin Miniprep Kit (Qiagen). For the Munc18a site-directed mutants (S158A, T574A), cDNA constructs were made by the polymerase chain reaction using specific oligonucleotide sense (amino acids 152–162, S158A; 566–586, T574A) and corresponding antisense primers. Oligonucleotide primers were synthesized by the University of Michigan DNA Core Facility. The construction of the Munc18a mutant cDNAs was confirmed by chain termination sequencing using the Sequenase version 2.0 DNA Sequencing Kit (Amersham Pharmacia Biotech).

The *Bam*HI and *Eco*RI fragment cut from GST-p25 was inserted into the *Bam*HI and *Eco*RI site of the vector pcDNA in which a Kozak sequence had been inserted to allow mammalian expression of the p25 cDNA under the control of the cDNA promoter.

Expression and Purification of Recombinant Proteins—Recombinant glutathione *S*-transferase (GST) fusion proteins were expressed in *E. coli* and subsequently purified by means of their affinity for glutathione-conjugated Sepharose 4B beads (Amersham Pharmacia Biotech) as described (31). Expression of recombinant proteins was induced by treatment with 0.2 mM isopropyl-1-thio-b-D-galactopyranoside (Boehringer Mannheim) for 4 h at 37 °C. The bacteria were lysed by treatment with a French cell press (1000 pounds/square inch pressure differential) and subsequently with 1% Triton X-100 for 1 h at 4 °C. When necessary, cleavage of the GST moiety from the fusion protein was accomplished by treatment with human thrombin (Sigma) at 0.2 NIH units/μl for 16 h at 20 °C. Alternatively, the entire GST fusion protein was eluted from the Sepharose 4B beads by treatment with 10 mM glutathione for 15 min at 20 °C. Protein production and purification was confirmed by Coomassie Blue staining and Western blotting.

Translocation and Cdk5 Activity Measurements—The cytosolic *versus* particulate distribution of Cdk5 was examined in cell or tissue samples under control conditions or following exposure to membrane-depolarizing stimuli. Control physiological saline contained 40 mM NaCl, 100 mM *N*-methyl-D-glucamine-Cl, 5 mM $KHCO_3$, 2.2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES (pH 7.2). Elevated potassium saline solutions were prepared by appropriate addition of KCl (50 or 100 mM), concomitant with an equivalent reduction in concentration of *N*-methyl-D-glucamine-Cl. Calcium-free solutions omitted $CaCl_2$ and included 1 mM EGTA. Following treatment, cells were lysed in buffer containing 2 mM EDTA, 2.25 mM β-glycerol phosphate, 20 mM Tris (pH 7.5), 175 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 50 μM olomoucine. Lysates were then centrifuged at 100,000 × *g* for 25 min at 4 °C. The supernatant constituted the cytosolic fraction. The resulting pellet was suspended in lysis buffer containing 0.2% Triton X-100, sonicated 3 × 5 s with 1-min intervals between each sonication and then re-centrifuged at 20,000 × *g* for 25 min at 4 °C. The resulting supernatant was the particulate fraction. Cdk5 content in each fraction was measured by SDS-PAGE, Western blotting, and probing for Cdk5 immunoreactivity by ECL detection. Visualization and quantitation of the signal was performed off both x-ray film and a GS-250 Molecular Imager. Cdk5 kinase activity was determined as described previously using a Cdc2 kinase assay kit (Upstate Biotechnology Inc.) by following incorporation of ^{32}P radiotracer into a histone H1 peptide from bovine calf thymus containing a predicted Cdc2 phosphorylation site. Specificity of the reaction to Cdk5 kinase activity was tested by inclusion of supplied peptide inhibitors of protein kinase C and protein kinase A within the reaction mixtures, together with an inhibitor of calmodulin-dependent protein kinase (R24571; 5 μM). In addition, kinase activity was tested for sensitivity to the Cdk5 inhibitor olomoucine or the much less active analogue iso-olomoucine.

Munc18a and mutant Munc18a phosphorylation reactions were performed by using Cdk5 immunoprecipitated from rat brain. Cdk5 immunoprecipitate was prepared by homogenizing rat brain in lysis buffer and spinning the resulting lysate at 30,000 × *g* for 30 min at 4 °C. The supernatant was collected and precleared with protein A-linked agarose beads for 1 h at 4 °C. Aliquots (1 ml) of the precleared supernatant were then treated for 1 h at 4 °C with 2 μl of anti-Cdk5 and subsequently with 100 μl of protein A-linked agarose beads for 1 h at 4 °C. The agarose beads were gathered by centrifugation and washed 3 times with phosphorylation buffer (50 mM Tris-HCl (pH 8.0), 1 mM EGTA, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM dithiothreitol). 30 μl of the beads (approximately 20 ng of Cdk5 immunoprecipitate) were then added to 300 μl of phosphorylation buffer containing ATP at 0.5 mM and substrate (*i.e.* wild type and mutant Munc18s) at 1 μM. The reaction mixtures were prepared and kept at 4 °C to prevent the start of the phosphorylation reactions. Olomoucine and iso-olomoucine were also added in 50 and 200 μM concentrations, respectively, to certain reactions. The reactions mixtures were kept at 4 °C and then spiked with 3 μl (30 μCi) of [γ-^{32}P]ATP and incubated for 30 min at 30 °C. For kinase assay quantification, an aliquot of the reaction mixture was blotted onto phosphocellulose paper, washed with 0.75% phosphoric acid, and the incorporated radioactivity determined by liquid scintillation counting. Specificity of ^{32}P incorporation into wild type or mutant Munc18a was determined by subjecting aliquots of each reaction mixture to SDS-PAGE followed by visualization and quantitation with a GS-250 Molecular Imager (Bio-Rad).

Determination of Fusion Protein Interactions and Analysis of Regulation by Cdk5—Binding relations between Munc18a or mutant Munc18s and syntaxin 1a were performed by incubation of GST-Munc18a proteins at 300 nM (600 nM for Munc18a T574A) bound to glutathione-Sepharose 4B beads with given concentrations of syntaxin 1a in binding buffer. Binding buffer contained 4 mM HEPES/NaOH (pH 7.4), 0.1 M NaCl, 1 mM EDTA, 3.5 mM $CaCl_2$, 3.5 mM $MgCl_2$. After overnight incubation with rotation at 4 °C, samples were centrifuged, washed extensively, and pellets resuspended in SDS-sample buffer. Analysis of syntaxin 1a binding was determined by SDS-PAGE of each sample, followed by Western blotting and probing for syntaxin 1a immunoreactivity by ECL. Quantitation of the signal was performed by phosphorimaging.

For kinase-induced protein dissociation studies, the Munc18a·syntaxin 1a heterodimer complex was formed by incubating 12 μg of Munc18a (either wild type or mutant) with 50 μg of GST-syntaxin bound to glutathione-Sepharose 4B in 300 μl of protein binding buffer for 1 h at 4 °C. The Sepharose beads were then pelleted and washed extensively with protein binding buffer to remove all the unbound Munc18a. Next, the complex was eluted off the purified Sepharose 4B beads by treatment with 100 μl of 10 mM glutathione for 15 min at 20 °C. The supernatant containing the eluted complex was then injected into a 10K Dialysis Cassette (Pierce) and incubated in 750 ml of phosphorylation buffer with constant stirring for 2 h at 4 °C in order to remove the glutathione. The Munc18a·GST·syntaxin 1a complex was then recovered from the cassette, and aliquots of approximately 15 μg of total protein were added to 300 μl of phosphorylation buffer containing 0.5 mM ATP and either Cdk5 immunoprecipitated from rat brain lysate or 0.42 μg/ml protein kinase C (the protein kinase C reaction was conducted in the presence of 100 μM $CaCl_2$, 83.3 μg/ml phosphatidylserine, and 8.3 μg/ml diglyceride). Immunoprecipitated Cdk5 rather than bacterially expressed recombinant p25/Cdk5 protein was utilized for these experiments as it demonstrated higher specific catalytic activity, represented mammalian expressed Cdk5 protein, and could be more rapidly prepared. The reactions were incubated for 30 min at 30 °C with constant agitation, following which the Cdk5 immunoprecipitate on agarose beads was centrifuged. The supernatant was then mixed with 100 μl of glutathione-Sepharose 4B beads for 1 h at 4 °C to bind and subsequently pellet all the syntaxin 1a by centrifugation. The obtained pellet was washed 3 times with phosphorylation buffer. Aliquots of the pellet and supernatant from each sample were subject to SDS-PAGE, Western blotted, and probed for Munc18a immunoreactivity by ECL. Visualization of the signal was by both x-ray film and a GS-250 Molecular Imager.

Cell Preparation, Transfection, and Secretion Experiments—Chromaffin cell preparation, transient transfection, and secretion experiments were performed as described previously (32). For intact chromaffin cells, secretion experiments were performed in a physiological salt solution containing 145 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 15 mM HEPES (pH 7.4), and 0.5 mM ascorbate. Secretion from digitonin-permeabilized cells was conducted in potassium glutamate solution containing 139 mM potassium glutamate, 20 mM PIPES (pH 6.6), 2 mM MgATP, and 5 mM EGTA with no added Ca^{2+} (Ca^{2+}-free) or 5 mM EGTA buffered with calcium to set a free calcium concentration of 30 μM. In non-transfected chromaffin cells, secretion was investigated by preincubating the cells for 3 h in Dulbecco's modified Eagle's medium/Ham's F-12 containing 10% heat-inactivated fetal calf serum, [^{3}H]norepinephrine, and 0.5 mM ascorbate. Cultures were rinsed for at least 30 min in medium without added [^{3}H]norepinephrine before inducing release with the nicotinic acetylcholine receptor agonist 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP). Analysis of release from transfected cells was carried out by measurement of human growth hormone that was co-transfected with the test vector (*i.e.* pcDNA-p25). Human growth hormone appearing in the medium was measured with a luminometric assay kit from Nichols Institute (San Juan Capistrano, CA).

Electrophysiology—Whole-cell patch clamp methods were used to evoke and record Ca^{2+} currents and measure the changes in membrane capacitance (ΔCm) from single bovine chromaffin cells using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Patch pipettes were constructed out of 1.5-mm outer diameter capillary glass (AM Systems), coated with Sylgard elastomer (Dow Corning, Midland MI), and fire-polished. The patch pipettes had tip resistances of 2–5 megohms and were filled with a pipette solution that contained 140 mM $CsMeSO_3$, 1 mM $MgCl_2$, 0.25 mM EGTA, 2 mM ATP, 0.5 mM Li-GTP, and 10 mM HEPES with pH adjusted to 7.1 with NaOH. For recording, the cells were placed in a solution containing 130 mM tetraethylammonium chloride, 10 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose with pH adjusted to 7.15 with Tris. The whole-cell capacitance and 60–70% of the series resistance were compensated electronically. High resolution measurements of changes in membrane capacitance reflecting net of exocytotic and endocytotic activity were performed using a modified phase-tracking method with a software-based (Pulse Control) phase-sensitive detector (33). A 19.1-kHz sampling rate was used to compute 1 Cm point for each 13 ms. Calibration pulses of 100 femtofarads and 500 kΩ were generated and placed at the beginning of each Cm data record.

RESULTS

Cdk5 Regulation of Munc18a-Syntaxin 1a Interaction—To demonstrate that Cdk5 was capable of phosphorylating Munc18a, Cdk5 was immunoprecipitated from rat brain lysate and incubated in a [^{32}P]ATP solution with recombinant Munc18a fusion protein. The obtained autoradiograph shows that immunoprecipitated Cdk5 causes ^{32}P incorporation into Munc18a (Fig. 1*A*). A 23-kDa upward shift of the radiolabeled signal in the GST-Munc18a sample corresponds to the additional mass of the GST moiety and verifies that Munc18a is the substrate for this reaction. That Cdk5 is the phosphorylating kinase is demonstrated by inhibition of phosphate incorporation by the specific Cdk inhibitor olomoucine (50 μM). Olomoucine, a purine analogue, has been demonstrated to exhibit little or no inhibition of many other protein kinases including protein kinase C, protein kinase A, and protein tyrosine kinases (34). As a control, protein A-linked agarose beads which had been incubated with the rat brain lysate for a commensurate period, but without pretreatment of the lysate with Cdk5 antibody, failed to demonstrate radiotracer incorporation.

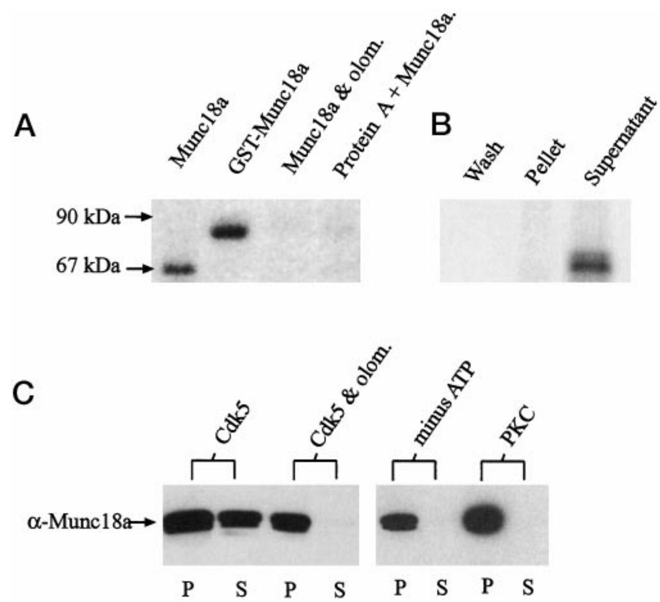

FIG. 1. **Cdk5 phosphorylation of Munc18a and effects on assembled Munc18a·syntaxin heterodimer complex.** *A*, Cdk5 immunoprecipitated from rat brain lysate was incubated with bacterially expressed and purified Munc18a or GST-Munc18a in phosphorylation buffer containing [γ-^{32}P]ATP at 30 °C for 30 min. After centrifugation, equivalent amounts of the supernatants were subjected to SDS-PAGE and autoradiography. Control reactions included 50 μM olomoucine or protein A beads that had been incubated with rat brain lysate in the absence of the immunoprecipitating Cdk5 antibody. *B*, autoradiograph demonstrating radiolabeling of Munc18a, which was prebound to GST-syntaxin 1a prior to incubation with immunoprecipitated Cdk5 and [γ-^{32}P]ATP. Following kinase treatment GST-syntaxin 1a and Munc18a remaining bound were pelleted by centrifugation after addition of glutathione-Sepharose beads. Munc18a appearing in the supernatant was radiolabeled. *C*, preformed Munc18a·GST·syntaxin 1a heterodimer complexes were incubated with Cdk5 immunoprecipitated from rat brain lysate or with purified protein kinase C in appropriate phosphorylation buffers containing ATP. Samples were then centrifuged and pellets washed extensively. Equivalent aliquots of the pellet and supernatant of each sample were then subjected to SDS-PAGE, Western-blotted, and probed for Munc18a immunoreactivity. Samples containing olomoucine (50 μM) or where ATP was omitted were included as kinase activity controls.

To determine whether Cdk5 could phosphorylate Munc18a when bound to syntaxin 1a, we utilized a pre-assembled Munc18a·GST·syntaxin 1a heterodimer complex that was subsequently incubated with Cdk5 immunoprecipitate in a [^{32}P]ATP-containing solution. Following incubation, the GST-syntaxin 1a was pelleted by centrifugation following addition of Sepharose 4B-glutathione beads and then washed extensively. The GST-syntaxin 1a pellet and the supernatant from the reaction were then probed for Munc18a radiolabeling. The autoradiograph showed labeling of a 67-kDa protein in the supernatant fraction alone, demonstrating that Munc18a bound to syntaxin is a substrate for Cdk5 phosphorylation and that phosphorylation induces disassembly of the complex (Fig. 1*B*). To determine the extent of Munc18a dissociation from GST-syntaxin 1a, the experiments were repeated and the fractions

TABLE I
Comparison of Cdk5 consensus phosphorylation sequences across Munc18a homologues

Amino acid residues in brackets correspond to the two consensus sequences in Munc18a for Cdk5. Underlined residues indicate presence of key amino acid residues.

Protein	Homologue with Munc18a	Organism	Tissue	S158 Site	T574 Site
	%				
Sec1p	27	*S. cerevisiae*	N/A	EE[EDAR]NG	IL[TPTK]FL
Unc-18	59	*C. elegans*	Ubiquitous	YY[NAQK]QG	II[TPDK]FL
Rop	63	*Drosophila*	Ubiquitous	LY[SPAF]AS	IL[SPEL]FL
nSec1[a]	100	Rat	Brain	FY[SPHK]AQ	IL[TPQK]LL
Munc-18b	63	Mouse	Kidney, testes, liver	PF[RAGE]RG	IL[TPTR]FL
Munc-18c	52	Mouse	Ubiquitous	CY[SPDP]SN	IL[TPRK]LL
s-Sec1	66	Squid	Stellate ganglion	YY[NPSR]AQ	IL[TPEG]LL

analyzed for Munc18a by immunoblotting. The resulting immunoblot reveals that conditions supporting Cdk5 kinase activity induce considerable dissociation (approximately 30–50%) of Munc18a from syntaxin 1a (Fig. 1*C*). This dissociation was found to be ATP-dependent and olomoucine-sensitive. In addition, comparable dissociation could not be achieved by protein kinase C, another putative regulator of the Munc18a·syntaxin 1a complex.

The Munc18a amino acid sequence possess two consensus phosphorylation sequences for Cdk5 at residues 158–161 (SPHK) and residues 574–577 (TPQK). Analysis of Munc18a homologues revealed a high degree of preservation of the Cdk5 phosphorylation sequence, which includes the Thr^{574} residue but not that of the Ser^{158} residue (Table I). To determine the importance of the Ser^{158} and the Thr^{574} sites to the Cdk5-induced phosphorylation and subsequent dissociation of Munc18a binding from syntaxin 1a, single site directed mutations (alanine substitution) of each site were generated (S158A and T574A). Initially, the S158A and T574A Munc18a mutants along with the wild type Munc18a were tested in a kinase assay as substrates for ^{32}P incorporation by Cdk5 immunoprecipitated from rat brain lysate (Fig. 2*A*). The Thr^{574} mutant failed to act as substrate for Cdk5, whereas the S158A mutant served in a manner statistically indistinguishable from the wild type Munc18a (6.3 ± 0.4% for T574A *versus* 90 ± 9% for S158A *versus* 100 ± 10% for wild type Munc18a; $n = 6$). To verify that the radiotracer incorporated was within the Munc18a protein, additional reactions were run, and the protein was then separated by SDS-PAGE. As shown by the resulting autoradiograph, although the S158A mutant was phosphorylated similarly to the wild type protein, the T574A mutant showed incorporation at a level no higher than the background control (Fig. 2*B*). The GST-Munc18a and 50 μM olomoucine controls demonstrated the substrate and kinase specificity of the reactions. An iso-olomoucine (200 μM) control was also included to demonstrate the specificity of olomoucine.

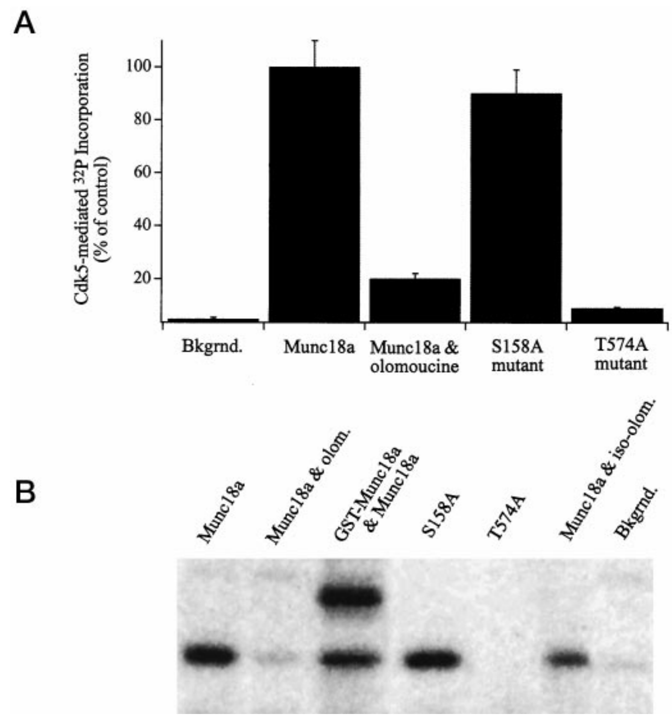

FIG. 2. **Effect of site-directed mutations of Munc18a on Cdk5-mediated phosphate incorporation.** *A*, recombinant Munc18a and mutant Munc18a protein were incubated with Cdk5 immunoprecipitated from rat brain in [γ-^{32}P]ATP containing phosphorylation buffer. Quantification of radiotracer incorporated was performed as described under "Experimental Procedures." Background reaction (*Bkgrnd.*) contains immunoprecipitated Cdk5 with no Munc18a. Inclusion of olomoucine (50 μM) with Munc18a demonstrates specificity of ^{32}P incorporation to Cdk5 activity. *B*, specificity of radiotracer incorporation into Munc18a and mutant Munc18a protein. Aliquots of phosphorylation reactions performed as in *A* were subjected to SDS-PAGE followed by visualization with phosphorimaging. Olomoucine (*Olom.*)- (50 μM) and iso-olomoucine (*Iso-olom.*) (200 μM)-treated reactions were included as controls for kinase specificity.

A set of further experiments attempted to determine whether phosphorylation at the Thr^{574} residue was responsible for the Cdk5 induced dissociation of Munc18a from syntaxin 1a. First, we examined the effect of the S158A and T574A mutations on recognition by the Munc18a antibody and on binding to GST-syntaxin 1a. Western blots of wild type *versus* the mutant Munc18a-expressed proteins showed no effect of the mutations on the strength of the immunoreactive signal. To evaluate protein interactions, Munc18a or mutant Munc18s containing the GST moiety were incubated with syntaxin 1a. Binding was then determined by collection and extensive washing of the glutathione Sepharose 4B beads to which GST bound followed by elution and analysis by SDS-PAGE and Western blotting of bound syntaxin 1a. Binding of syntaxin 1a was found to be saturable for each Munc18a protein construct, and although the T574A Munc18a construct showed approximately 20-fold less total binding than the wild type or S158A Munc18a protein, no significant difference was found in the 50% effective concentration (EC_{50}) for binding of the three Munc18a proteins (Fig. 3*A*). Next, to assess the ability of the kinase to induce dissociation of the heterodimer complexes, both the wild type and mutant Munc18a proteins were bound to GST-syntaxin 1a and treated with immunoprecipitated Cdk5 in an ATP-containing solution. The GST-syntaxin 1a was pelleted, washed thoroughly, and, along with the retained supernatant, probed for Munc18a immunoreactivity. The obtained immunoblot revealed that whereas Cdk5 was capable of inducing dissociation of both wild type and the S158A mutant from syntaxin 1a, it could not disassemble the T574A mutant from syntaxin 1a (Fig.

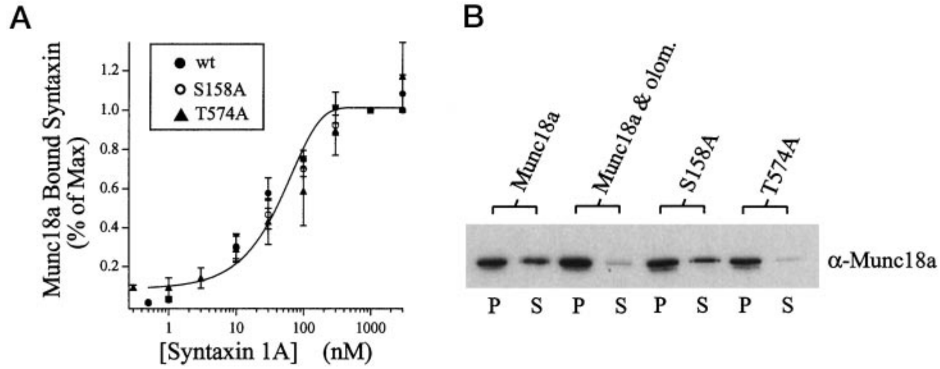

FIG. 3. **Binding of recombinant Munc18a and mutant Munc18a protein to syntaxin 1a and effect of Cdk5 phosphorylation on disassembly of resulting heterodimer complexes.** *A*, Munc18a or mutant Munc18a recombinant GST fusion proteins (300 nM except Munc18a T574A, which was 600 nM) linked to glutathione-Sepharose 4B beads were incubated in binding buffer with given concentrations of syntaxin 1a. After incubation, the samples were centrifuged, pellets washed, and then subjected to SDS-PAGE followed by Western blotting. Blots were probed for syntaxin 1a immunoreactivity, and the signal was quantitated by phosphorimaging. Syntaxin 1a bound to the recombinant Munc18a proteins was normalized for each reaction as a percent of the signal obtained at a saturating value of 1 μM syntaxin 1a ($\bar{x} \pm$ S.E. for each point, $n = 4$ wild type Munc18a; $n = 3$ S158A and T574A). *Solid line* represents least squares fit of combined data. *B*, preformed Munc18a-GST or mutant Munc18a protein-syntaxin 1a heterodimer complexes were incubated with Cdk5 immunoprecipitated from rat brain lysate in phosphorylation buffers containing ATP. Following centrifugation, equivalent aliquots of pellet and supernatant fractions of each sample were subjected to SDS-PAGE, Western-blotted. and probed for Munc18a immunoreactivity. Samples containing olomoucine (50 μM) were included as kinase activity controls.

3*B*). A reaction containing olomoucine with wild type Munc18a run as a control further demonstrated the specificity of the reaction to Cdk5 activity.

Calcium-dependent Cdk5 Translocation and Activation—Dynamic regulation of secretory activity by Cdk5 necessitates that the kinase itself be strictly regulated. As secretion from excitable cells is triggered by membrane depolarization and activation of Ca^{2+} influx, these effects on Cdk5 activity were investigated on neuroendocrine nerve endings isolated from the rat pituitary neural lobe, as well as on bovine adrenal chromaffin cells and on the neuroendocrine PC-12 cell line. Membrane depolarization with elevated extracellular concentrations of K^+ was found in each case to induce translocation of Cdk5 from a cytosolic to a particulate cellular compartment (Fig. 4*A*) and to be accompanied by an activation of Cdk5 kinase activity (Fig. 4*B*). The translocation resulted in approximately a doubling of particulate Cdk5 content. In addition, the depolarization-induced Cdk5 translocation was observed to be Ca^{2+}-dependent in the nerve endings and PC-12 cells, while considerable variation was observed for this parameter for chromaffin cells. The variation in chromaffin cells may result from a higher degree of cell heterogeneity retained in the isolation and culture of these cells. The enhancement of Cdk5 kinase activity was found in each of the cell preparations, however, to be dependent on the presence of extracellular Ca^{2+} during the period of membrane depolarization.

Cdk5 Regulation of Neuroendocrine Secretion—Since conditions that activate cellular secretion were observed to alter Cdk5 translocation and Cdk5 kinase activity, the effects of Cdk5 activity on neuroendocrine secretion from chromaffin cells were investigated. Cells were exposed to the Cdk inhibitor olomoucine, the less active analogue iso-olomoucine or the drug carrier (Me_2SO) in culture medium for 16 h prior to stimulation of [^{3}H]norepinephrine secretion by the nicotinic acetylcholine receptor agonist DMPP. Olomoucine treatment resulted in an average 30% decrease in DMPP-stimulated secretion with respect to that of control (Fig. 5*A*). No significant effects of either olomoucine or iso-olomoucine were observed on basal (*i.e.* non-stimulated) [^{3}H]norepinephrine secretion. Secretion was also investigated on digitonin-permeabilized chromaffin cells to evaluate further the effects of Cdk5 inhibition on secretion and to determine if Cdk5 alters the secretory response after Ca^{2+} influx. Permeabilization of the cells was carried out under low calcium conditions (5 mM EGTA) in the presence of the Cdk5 inhibitor or its analogue, and secretion was subsequently stimulated with a free Ca^{2+} concentration of 30 μM. Olomoucine exhibited a dose-dependent inhibition of Ca^{2+}-induced secretion with respect to the iso-olomoucine control (Fig. 5*B*). At 300 μM, olomoucine inhibition averaged 28%. Iso-olomoucine itself demonstrated no inhibitory effects on secretion over the concentration range tested. As Ca^{2+} has free access to the cell interior in digitonin-permeabilized cells, the observed inhibition of secretion by olomoucine suggests that Cdk5 action on secretion is not via alteration of Ca^{2+} influx.

The effects of Cdk5 inhibition on secretion were examined further at the single chromaffin cell level under whole-cell voltage clamp. Membrane capacitance changes were measured in response to depolarizing stimuli to provide highly time-resolved measurements of exocytotic and endocytotic activity and to evaluate changes in Ca^{2+} sensitivity of secretion. Repetitive depolarization of the membrane from a holding potential of -90 mV to a step potential of $+20$ mV (50-ms duration, 200-ms interpulse interval) was used to activate voltage-dependent calcium channels and allow Ca^{2+} influx. This stimulation resulted in a rapid increase in membrane capacitance which was followed by a slow recovery on cessation of stimulation. Representative data comparing changes in membrane capacitance for an olomoucine and an iso-olomoucine-treated cell are shown in Fig. 6*A*. Olomoucine inhibited the stimulated membrane capacitance increase, despite a very similar level of time-integrated evoked Ca^{2+} influx between the cells (Fig. 6*B*). Furthermore, the olomoucine inhibition of secretory responses was observed over a range of Ca^{2+} influx values, with differences most prominent in cells that demonstrated higher influx (Fig. 6*C*). Averaged changes in membrane capacitance normalized to total time-integrated Ca^{2+} influx (femtofarads/picocoulombs) under control conditions ($n = 4$) or following iso-olomoucine ($n = 8$) and olomoucine ($n = 8$) treatment are shown in Fig. 6*D*. Chromaffin cells treated with olomoucine gave significantly ($p < 0.5$, $n = 8$) smaller stimulated increases in membrane capacitance than iso-olomoucine-treated cells.

Cdk5, like other members of the family of cyclin-dependent kinases, are not active as monomeric proteins but rather require binding of specific proteins to form an active heterodimeric holoenzyme (14, 15). Although cyclins represent the

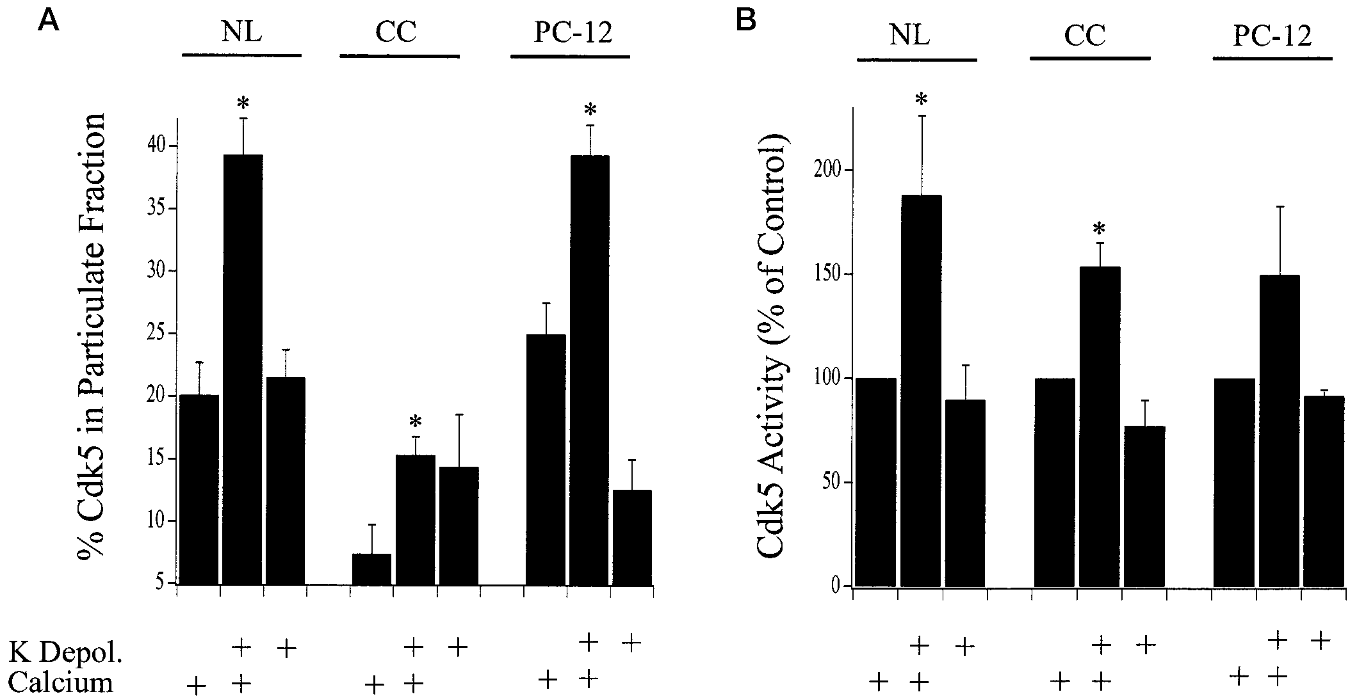

FIG. 4. **Translocation and activation of Cdk5 by a secretory stimulus.** *A*, Cdk5 protein present in cytosolic and particulate fractions was determined from quantitation of immunoblots following SDS-PAGE under control (Ca^{2+}-containing physiological saline) and membrane-depolarizing (*Depol.*) conditions (elevated extracellular $[K^+] \pm [Ca^{2+}]_o$). Determinations were performed for isolated nerve endings from the neural lobe of the pituitary (*NL*), from primary cultures of bovine chromaffin cells (*CC*), and from PC-12 cells (*PC-12*). Depolarizing treatments were as follows: neural lobe, 100 mM $[K^+]$, 10 min; chromaffin cells and PC-12, 50 mM $[K^+]$, 5 min. Results are expressed as a percentage of the total Cdk5 protein present in the particulate fraction ($\bar{x} \pm$ S.E.; neural lobe, $n = 11$; chromaffin cells, $n = 6$; PC-12 cells, $n = 3$). *B*, Cdk5 kinase activity of neural lobe (*NL*), chromaffin cells (*CC*), and PC-12 cells were analyzed following indicated treatments (as in *A*). Kinase activity is given as a percentage of the activity ($\bar{x} \pm$ S.E., neural lobe, $n = 4$; chromaffin cells, $n = 6$; and PC-12 cells, $n = 3$) determined under control conditions for each preparation. *Asterisks* indicate significant difference ($p < 0.05$) from control in *A* and *B* by Student's *t* test or Wilcoxon signed rank test.

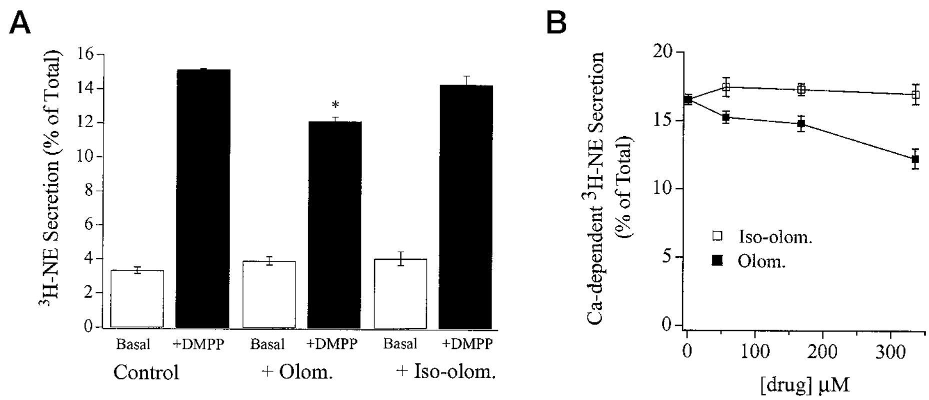

FIG. 5. **Effects of Cdk5 inhibitor olomoucine on [^{3}H]norepinephrine secretion from intact (*A*) and permeabilized (*B*) bovine chromaffin cells.** Chromaffin cells were preloaded with [^{3}H]norepinephrine for 3 h, rinsed, and incubated a further 30 min prior to measurement of basal and evoked [^{3}H]norepinephrine release. Release responses were evoked from intact cells by exposure to the nicotinic acetylcholine receptor agonist DMPP (20 μM) for 4 min and from permeabilized cells by raising the free Ca^{2+} concentration of the medium to 30 μM for 15 min. Intact cells were pretreated with olomoucine (*Olom.*, 167 μM), iso-olomoucine (*Iso-olom.*, 167 μM), or drug carrier (*i.e.* Me_2SO 0.5%, control) for 16 h in Dulbecco's modified Eagle's medium, and the drug concentrations were maintained throughout the [^{3}H]norepinephrine loading and release portions of the experiments. In each experiment $n = 3$ wells/group A, and *asterisk* indicates significant difference ($p < 0.05$) for olomoucine *versus* control; *B*, $p < 0.05$ for olomoucine *versus* iso-olomoucine at concentrations greater than 100 μM.

activator of most Cdks, neural Cdk5 is activated by a brain-specific protein termed p35 (also termed p35^{nck5a}) that is highly expressed in post-mitotic neurons and a p35 proteolytic cleavage product termed p25 (19, 35, 36). To determine whether p35/Cdk5 kinase activity participates in regulation of neurosecretion, we altered the endogenous levels of p25 in chromaffin cells by transfection with plasmid DNAs. Analysis of release from transfected cells was carried out by measurement of human growth hormone, which was expressed in the chromaffin cells by co-transfection of a hGH vector with the p25 expression vector. Initial investigations confirmed that transfection and expression of the p25 protein in HEK 293 cells led to greatly increased (>10-fold) Cdk5 kinase activity. Assessment of the effect of p25 transfection and expression on Cdk5 activity in primary cultures of chromaffin cells was precluded by low transfection efficiency (approximately 1–2%). In two experiments, DMPP-induced hGH secretion was increased by 45 ± 2% following transfection and expression of p25, over the control pCMV neo-transfected chromaffin cells. No effect of p25 was observed on basal secretion. Thus, the results with the p25 transfection support an important function for Cdk5 in the secretory mechanism.

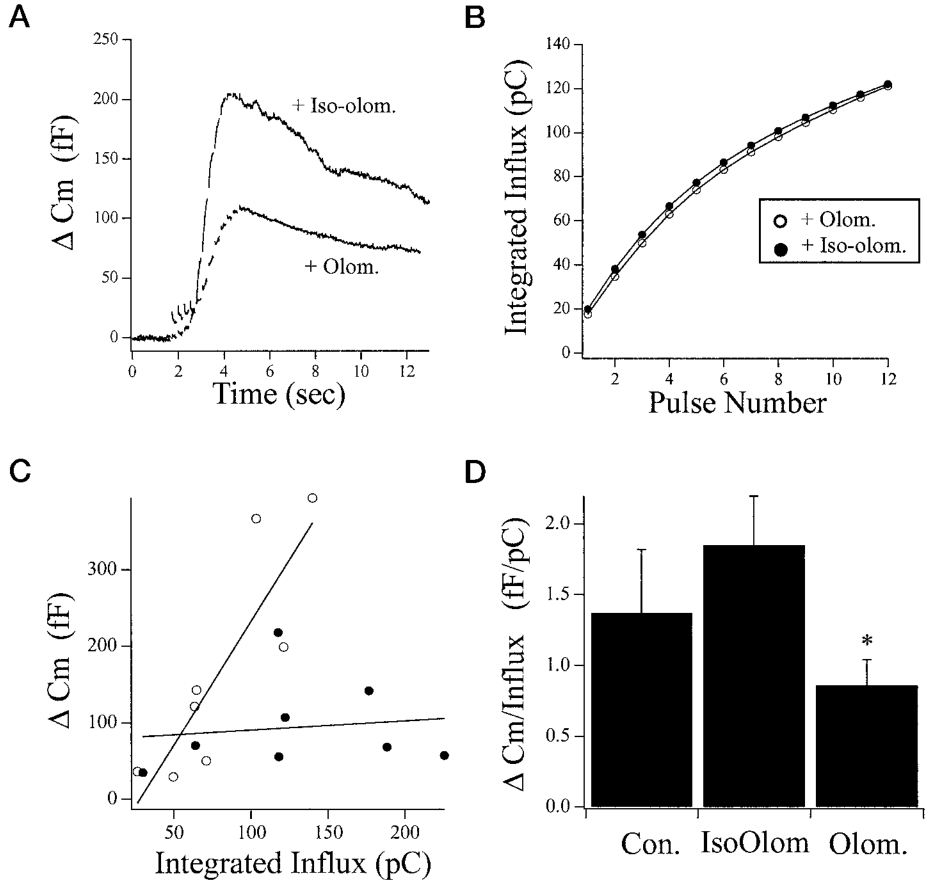

FIG. 6. **Effects of olomoucine on temporally resolved membrane capacitance changes (ΔCm) evoked by membrane depolarization under voltage clamp of single chromaffin cells.** *A*, representative ΔCm of chromaffin cells incubated with olomoucine (*Olom.*) or iso-olomoucine (*Iso-olom.,* 167 μM, 16 h) in response to 12 repetitive step depolarizations (−90 mV holding potential to +10 step potential for 50 ms repeated at 5 Hz). Breaks in records indicate periods of membrane depolarization. *B*, cumulative time-integrated Ca^{2+} influx in response to the step depolarizations of the records shown in *A*. *C*, relationship of the total time-integrated Ca^{2+} influx in response to repetitive step depolarizations (as in *A*) to the peak ΔCm for olomoucine- (*filled symbols*) and iso-olomoucine (*open symbols*)-treated cells (167 μM, 16 h). Each value represents a determination from a separate cell in response to the first series of applied step depolarizations. *D*, ΔCm normalized to total integrated Ca^{2+} influx ($\bar{x} \pm$ S.E.) for control ($n = 3$), iso-olomoucine- ($n = 8$), and olomoucine ($n = 8$)-treated chromaffin cells. $p < 0.05$ for olomoucine *versus* iso-olomoucine, Student's *t* test.

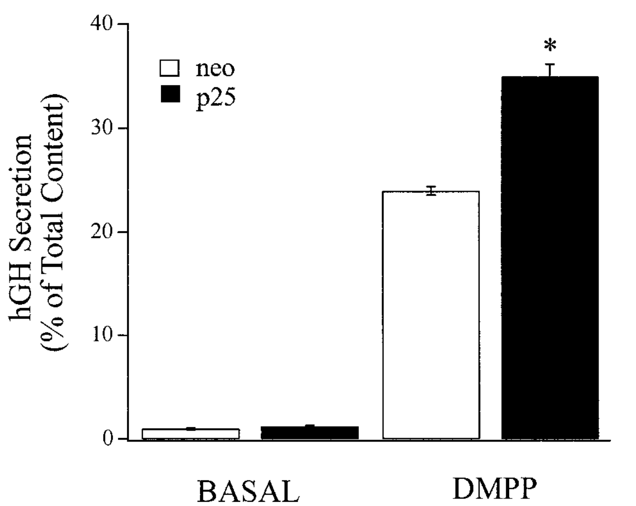

FIG. 7. **Transient transfection with plasmid encoding p25 increases secretion of co-transfected and expressed growth hormone (*hGH*).** hGH secretion was determined from transfected chromaffin cells that were co-transfected with a plasmid encoding either bovine p25 or a parent pCMV plasmid (*neo*). Basal and DMPP (10 μM, 5 min)-induced secretions of hGH were determined 4–6 days following transfection. In each experiment $n = 4$ wells/group. $p < 0.05$ for DMPP stimulated secretion of hGH from p25 *versus* neo.

DISCUSSION

It has previously been shown that Munc18a copurifies via a direct protein-protein interaction with Cdk5 from rat spinal cord (14). We previously reported that Munc18a is a phosphoprotein *in situ* and that it is subject to phosphorylation by Cdk5 *in vitro* (30). The present report demonstrates that a preformed Munc18a·syntaxin 1a heterodimer complex can be disassembled by addition of catalytically active Cdk5. We further identify a threonine residue 20 amino acids from the carboxyl terminus of Munc18a (Thr^{574}) as being the phosphorylation site. A conservative mutation of this site to alanine blocked both ^{32}P radiotracer labeling of Munc18a by Cdk5 *in vitro* and disassembly of the preformed Munc18a·syntaxin 1a complex. As the binding of Munc18a with syntaxin 1a requires the full length of Munc18a sequence and also requires the complete cytoplasmic domain of syntaxin 1a (37–39), a conformational or charge change caused in this region by the phosphorylation of Thr^{574} may underlie the normal disassembly of the Munc18a-syntaxin 1a heterodimer. A second putative phosphorylation site (Ser^{158}) defined by Cdk5 phosphorylation consensus sequence is not subject to phosphorylation *in vitro* by active Cdk5. Mutation of either site (Thr^{574} or Ser^{158}) had no significant effect on the EC_{50} for binding of Munc18a to syntaxin. Treatment of the Munc18a·syntaxin 1a complex with protein kinase C under conditions conducive to phosphorylation failed to affect the stability of the complex, suggesting that although protein kinase C can phosphorylate Munc18a that is not bound to syntaxin 1a, it cannot induce disassembly of the already formed heterodimer complex.

The identification of Thr^{574} as the phosphorylation site is consistent with the relatively high degree of conservation at the Thr^{574} site and the lack of conservation at the Ser^{158} site among Munc 18 homologues. In both Sec1p (9) and Unc-18 (40), which show 26 and 59% homology, respectively, at the amino acid level, the Thr^{574} site is 100% conserved, whereas the Ser^{158} site is not. Although these organisms do not have Cdk5, they do possess Cdc2, which recognizes the Thr^{574} site as a phosphorylation sequence and so could function similarly to Cdk5. A comparison of Munc18a to its mammalian homologues Munc18b and Munc18c (41) also point to importance of the Thr^{574} site. Munc18b, which is distributed in tissues other than brain, and Munc18c, which is distributed in brain as well as other tissues, again show conservation of the Thr^{574} but not the Ser^{158} site. In comparison, the *Drosophila* Munc18a homologue Rop (42) does not possess the (S/T)P*X*(K/R) sequence present in most of the other proteins. Although *Drosophila* Cdk5 shares 77% homology with human Cdk5, there are no reports of p35 in *Drosophila,* and the carboxyl sequence (SPEL) may be sufficiently preserved that it may be recognized by Cdk8, a Cdk unique to *Drosophila* (43). Therefore, it remains possible that in *Drosophila* both a Cdk and the carboxyl sequence of Rop are of importance to the process of secretion. Indeed, in the same way that the SNARE hypothesis holds in only a generalized sense across all membrane fusion events, the regulation of SNARE-interacting proteins may also be only generalized. For example, even though Munc18c possess the TP*X*K carboxyl sequence that would target it for Cdk5-induced disassembly from syntaxin 1a, Munc18c has only been found to bind syntaxin 5 (41). In addition, Munc18a has been reported to bind to other proteins including DOC2 (44) and MINTs (45) that are likely to affect Munc18a phosphorylation, as well as syntaxin 1a binding and function. An additional consideration is whether all the actions of Munc18a and its homologues are restricted to their interactions with the target SNARE syntaxins. Munc18a, SNAP-25, and syntaxin are not restricted to the synaptic region of neurons but are distributed throughout the axon and soma, suggesting the possibility of additional actions of Munc18a (46).

Substantial genetic and biochemical evidence exists to support an essential role of members of the Sec1 protein family in the secretory process. For example, SEC1 was identified as one of 10 genes in *S. cerevisiae* required for the final stages of secretion of protein to the cell exterior (9, 47); mutation in the *unc-18* gene in *Caenorhabditis elegans* caused abnormal accumulation of acetylcholine (40, 48), and Rop overexpression in *Drosophila* reduced spontaneous vesicle fusion and significantly decreased evoked responses to repetitive stimulation (49). Additional studies have demonstrated that loss of function mutations in the *Drosophila rop* gene results in a reduction of neurotransmitter release in adult photoreceptor cells (50) and that levels of Rop expression regulate evoked neurotransmission at the neuromuscular junction via an interaction with syntaxin (13). Yet the precise nature of the role of Munc18a in secretion is less clear. For example, exogenous addition of Munc18a to permeabilized chromaffin cells had no effect on calcium-induced secretion and overexpression of Munc18a in transiently transfected PC-12 cells did not affect the extent of evoked exocytosis (51). In contrast, microinjection of a squid neuronal homologue of Sec1 protein into the squid giant synapse inhibited evoked neurotransmitter release but did not alter the distribution of synaptic vesicles at active zones (52).

Another important consideration is whether Cdk5 regulation of the secretory pathway is itself regulated. That is whether Cdk5 phosphorylation of Munc18a is a persistent tonic affect or a dynamic component of the secretory response that modifies the level of vesicles available for priming. Our present data are largely supportive of the latter possibility. For example, we have demonstrated that Cdk5 translocates from a cytosolic to a particulate fraction in response to membrane depolarization and activation of calcium influx. Membrane depolarization alone is an insufficient stimulus for this translocation event. Furthermore, the data demonstrate that induction of translocation produces a corresponding increase in the level of Cdk5 kinase activity. This increase in activity occurs over a period of minutes and, thus, likely represents activation of existing Cdk5 rather than long term regulation by transcriptional/translational activation and an increase in the Cdk5 activator protein p35 or the active proteolytic cleavage product p25. Consistent with this observation, a marked increase in Cdk5 activity occurring over minutes in response to ischemic brain injury in rats has been reported, the increased Cdk5 activity being found to occur without a corresponding increase in Cdk5 protein levels (53).

The mechanism through which rapid regulation of Cdk5 may be achieved is unknown, although certain parallels to the regulated activation of other cell cycle kinases may be drawn. Other Cdks are regulated by binding of specific cyclins, by cellular accumulation of cyclin and Cdk protein, by the phosphorylation state of each of three sites on Cdks, and by direct binding of a number of inhibitory proteins (*e.g.* Kip1 and p21). Direct evidence to support these mechanisms in rapid Cdk5 regulation is, however, limited. For example, regulation of Cdk5 activity by phosphorylation is also poorly supported, although the regulatory phosphorylation sites Thr^{14} and Tyr^{15} of Cdc2 are entirely conserved in Cdk5, and $Thr^{160/161}$ is substituted by serine. Whereas phosphorylation/dephosphorylation of these sites may modulate Cdk5 activity *in vitro*, it certainly is not required, as activation of recombinant Cdk5 occurs upon binding recombinant neural specific activator p35 or p25 in the absence of other kinase or phosphatase activity (17–19, 35, 36). Rapid regulation of Cdk5 activity could occur by increased binding of free p35 to monomeric Cdk5, particularly as the generally low cytoplasmic levels of p35 (30, 54) are under strict regulation, with rapid p35 turnover (half-life 20–30 min) controlled by a ubiquitin-proteasome pathway (27). Moreover, a GTP-dependent association between p35 and Rac has been recently reported to which Cdk5 can complex and be catalytically activated (55). Interestingly, the present findings showing Cdk5 translocation and activation in chromaffin cells, PC-12 cells and isolated peptidergic nerve endings suggests a general conservation of a rapid regulatory mechanism across neuroendocrine cells.

Based on our findings that catalytically active Cdk5 can prompt disassembly of a Munc18a·syntaxin 1a complex *in vitro* through phosphorylation of Munc18a and that Cdk5 is activated by conditions that stimulate secretion, we have attempted to determine if the level of Cdk5 activity correlated to secretory responsiveness. The results provide evidence for the importance of Cdk5 activity in the secretory pathway, as pretreatment of intact chromaffin cells with the Cdk inhibitor olomoucine inhibited DMPP and membrane depolarization evoked secretion. The inhibition was not observed with the analogue iso-olomoucine, which differs from olomoucine only in the location of a methyl group on the imidazole ring of the purine backbone. The inhibitory effects of olomoucine were not mediated by effects on calcium influx. Overall, however, the secretory inhibition by olomoucine treatment, while statistically significant, was modest and thus allows several interpretive possibilities. For example, it is possible that the olomoucine treatment failed to inhibit completely Cdk5 activity, that Cdk5 phosphorylation of Munc18a is redundant with other mechanisms to disassemble the Munc18a-syntaxin heterodimer, or that assembly/disassembly of a Munc18a·syntaxin complex is not rate-limiting in acute secretory responses. To avoid potential problems of the specificity of the Cdk inhibitor olomoucine to experimental interpretation, we also transiently transfected and overexpressed the Cdk5 activator p25 in chromaffin cells and examined effects on evoked secretion. Overexpression of p25 dramatically increased evoked secretion consistent with increased Cdk5 activity leading to secretory effects.

The present data are supportive of a model whereby phosphorylation of Munc18a by Cdk5 mediates disassembly of preformed Munc18a·syntaxin 1a complexes. That these complexes form *in situ* is supported by genetic linkage of Sec-1 and Sso1p and Sso2p in yeast (56), by yeast two-hybrid screens for Munc18-interacting proteins (44), by genetic evidence in *Drosophila* (13), by immunocytochemical overlap of a portion of cellular Munc18 and syntaxin (46), and by repeated demonstration of high affinity binding of Munc18a with syntaxin 1a *in vitro* (8, 57). However, co-immunoprecipitation from cellular lysates has proved difficult, and both syntaxin 1a and Munc18 proteins show a distribution in neurons that includes axonal and somatic regions (46). Thus, although there may be additional functions for Munc18, genetic studies from yeast, *C. elegans,* and *Drosophila* clearly establish an essential requirement of Sec1 protein and its homologues to the secretory pathway. The Cdk5-mediated dissociation of Munc18a from syntaxin 1a may be important in making available competent sites for vesicle SNARE interaction with target SNAREs, as this SNARE interaction is blocked *in vitro* when Munc18 is bound to syntaxin. The rapid increase in the level of Cdk5 activity during secretory conditions suggests a mechanism by which the rate of SNARE interactions can be dynamically regulated and which would ultimately lead to changes in secretory responsiveness.

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